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The evolution of leaf anatomy in Badiera (Polygalaceae)

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The evolution of leaf anatomy in Badiera (Polygalaceae)

(TITLE)

BY

Laura Brauer

THESIS

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ABSTRACT

Leaf samples from Polygalaceae: nine species of *Badiera*, two species of *Bredemeyera*, three species of *Hebecarpa*, and two species of *Polygala* were taken from herbarium specimens for anatomical observations. Several methods of rehydration were compared to a control of fresh material fixed in the field for four species of *Badiera.* Soaking in concentrated ammonium hydroxide proved to be the best overall means of rehydrating leaves of the genus *Badiera* and related genera. All leaves were embedded in paraffin for sectioning and cleared in 5% aqueous sodium hydroxide for examining epidermal features. Twelve anatomical characters were mapped onto a molecular phylogeny to determine patterns of variation for the genus *Badiera* and related genera. The clade of *Badiera* + *Hebecarpa* is defined by having level hair bases and biseriate palisade mesophyll. Species of *Badiera* are united by a thick adaxial cuticle and revolute margins. Species of *Hebecarpa* are united by the presence of a thin adaxial cuticle, amphistomatal leaves, palisade mesophyll distributed isobilaterally, and a plane margin.

ACKNOWLEDGMENTS

As all wise people know, nothing of importance in life is done alone. With that sentiment foremost in my mind, I would like to acknowledge the people who played a role in my important achievement. Dr. J. Richard Abbott graciously allowed me access to his phenomenal specimen collection, patiently shared a vision, and judiciously decided what was worthy. Without him, this thesis would never have been started. Dr. Barbara Carlsward good-naturedly opened her lab and her heart to me, unwearyingly guided me every step of the way, and instinctively made me a better scientist and person. Without her, this thesis would never have been completed. Dr. Andrew Methven willingly addressed all my concerns, amazingly made everything work, and selflessly makes the MSNS program. Without him, the process would have been bumpy. Dr. Scott Meiners freely sat on my committee, and jokingly frightened me with questions he might ask during my defense. Without him, I would have been short a committee member. Ruth Atkins unselfishly helped me enter the Masters of Science in Natural Science program at Eastern Illinois University, bravely allowed me to be her roommate, and unwittingly helped me more than she will ever know. Without her, my degree would have ever happened. Vie Hodel and Sydney Hodel proudly watched me go through the master's process and kindly did not burn down the house. Without them, my life would have no purpose. Mark Siciliano magnanimously played the role of courier and confidant. Without him, the little things for my thesis would not have been accomplished. Ellen Stahr and Karen Donohoo tirelessly listened to me whine about specifics and life in general. Without these two friends, I would not be as close to sane as I appear. Scott Howard made me realize that a thesis would be worthwhile. Without him, I would not be as proud of the title of Masters of Science. Lastly, I would like to thank Eastern Illinois University, for giving me the resources to pursue my thesis. Without this institution and its staff, I would not have my master's degree and the additional opportunities that have and will come my way. Thank you – I did not achieve this alone.

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INTRODUCTION

Polygalaceae Hoffmans. & Link is a family of approximately 20 genera and 1000 species (Persson, 2001) distributed worldwide with a tremendous range in habit, from large trees and shrubs to small herbs and even mycoparasites (Abbott, 2009). *Badiera* DC. are shrubs 2 m or more in height that are geographically restricted to the Caribbean islands. Species of *Badiera* possess small green lateral sepals and thick-walled, dehiscent fruits that open to expose black seeds with orange arils that attract birds for dispersal (Abbott and Judd, 2011). Hairs found on the plant are simple and unicellular. Leaves are evergreen, dorsiventral, simple with an entire margin, and may contain glands. Stomata are amphistomatal or only abaxial in distribution and their configuration can be paracytic or anomocytic (Eriksen et al., 2000). Traditionally flower morphology has been used to classify genera of Polygalaceae, including *Badiera* (Persson, 2001). Chodat (1893) included *Badiera* as a subsection of *Hebecarpa* Chodat. Subsequent work, however, shows that *Hebecarpa* and *Badiera* are distinct from one another both ecologically and morphologically (Abbott and Judd, 2011). The present system of classification for Polygalaceae is polyphyletic (Abbott and Judd, 2011) and needs recircumscription (Persson, 2001). There are currently nine species of *Badiera* including the newly described *Badiera subrhombifolia* J.R. Abbott (Abbott and Judd, 2011).

There has been very little work on the vegetative anatomy of *Badiera*. In their treatment of dicotyledonous anatomy, Metcalfe and Chalk (1950) described the vegetative anatomy of Polygalaceae and only examined the wood anatomy of *Badiera*, which was not distinctive from other Polygaleae. Vessels ranged from small to large and solitary with simple perforation plates. Parenchyma distribution was mostly paratracheal, and rays were uniseriate and heterogeneous. Fibers had bordered pits and ranged in length from medium to short.

I examined and described the leaf anatomy of all nine species of *Badiera* in order to summarize the variation and address how such variation changes in an evolutionary context. For comparison, three species of *Hebecarpa*, two species of *Bredemeyera* Willd., and two closely related species of *Polygala* L. were included in my anatomical description and phylogenetic analyses.

Traditional rehydration techniques of plant material for anatomical study include boiling in water (Cutler, 1978; Beyers, 2000), boiling in water with a surfactant (Ayensu, 1967; Cutler, 1978; Keating, 2002), soaking in various concentrations of potassium hydroxide (Cunningham, 1969), and soaking in concentrated ammonium hydroxide (Toscano de Brito, 1996). Because my study relied heavily on the use of dried leaf material collected from herbarium specimens, I wanted to find the best rehydration method for leaves.

The goals of my thesis are: 1) determine the best rehydration methodology for leaf tissue of Polygalaceae; 2) describe the foliar anatomy of *Badiera* and related species; 3) document the pattern of evolutionary variation using the topology of a molecular phylogeny; and 4) document any shared-derived characters that define *Badiera* as a clade.

MATERIALS AND METHODS

Rehydration Techniques

To determine the best method of rehydration, leaf samples were taken from four species: *Badiera fuertesii* Urb., *Badiera oblongata* Britton, *Badiera subrhombifolia*, and

Badiera virgata Britton. Each sample was approximately 1 cm^2 including the midrib and margin from a standardized location in the center of the leaf (Cutler, 1978). *Badiera oblongata* leaves were too large to process as one piece, so the midrib and margin were processed as two separate samples. Leaf samples for each species were taken from one individual. Each species was rehydrated with three different rehydration methods: water with Aerosol OT, 3% potassium hydroxide, and 18.4 N ammonium hydroxide. Rehydration treatments were compared to a control of the same individual preserved from freshly collected material in FAA (0.5 part formalin: 0.5 part acetic acid: 9 parts ethyl alcohol) and stored in 70% ethyl alcohol.

The specimens treated with water and Aerosol OT were allowed to soak in 40 mL of deionized water with 6 drops of 10% Aerosol OT solution for 3 h. They were gradually warmed to boiling and boiled for 5-10 min until all pieces were no longer floating. The samples were rinsed with deionized water and stored in 70% ethyl alcohol.

Rehydration with potassium hydroxide was done at 65-70°C for approximately 50 min in 3% potassium hydroxide until all pieces were no longer floating. Samples were thoroughly rinsed with deionized water and stored in 70% ethyl alcohol.

Samples treated with 18.4 N ammonium hydroxide were placed in watch crystals and soaked for 20 h, until all pieces were no longer floating. The samples were thoroughly rinsed in deionized water and stored in 70% ethyl alcohol.

After treatment, all specimens and the control were dehydrated in a graded ethyl alcohol and tertiary butyl alcohol series, embedded in paraffin, and sectioned at 10 µm thickness on an American Optical 820 rotary microtome. Sections were attached to slides using Haupt's adhesive, stained with Heidenhain's iron-alum hematoxylin and safranin, and cover slips were affixed using Permount (Johansen, 1940). Observations were made using a Zeiss Axioskop 40 compound microscope and images were taken using a Pixera Pro 150ES digital camera.

Leaf Anatomy of *Badiera* and Related Genera

Leaf samples from nine species of *Badiera*, two species of *Bredemeyera*, three species of *Hebecarpa*, and two species of *Polygala* (Table 1) were rehydrated using the ammonium hydroxide method described above. For some species, I processed samples from more than one individual for a total of 29 samples. For cross sections, each specimen was embedded in paraffin and sectioned as described in the rehydration techniques section above.

Leaf clearings were made for each of the 29 samples described above by placing adaxial and abaxial pieces, which were approximately 1 cm^2 and contained the margin but no midrib, into deionized water until they sank. Samples were then placed in watch crystals containing a 5% aqueous sodium hydroxide solution for approximately 3 h at room temperature and rinsed with deionized water. Samples were soaked in bleach for 5-15 min to remove any remaining chlorophyll and thoroughly rinsed with deionized water. Samples were stained overnight at room temperature in watch crystals containing distilled water and 1-2 mL of 1% safranin in 50% ethanol. After staining, samples were dehydrated in graded ethyl alcohol series, cleared in limonene, and mounted in Canada balsam.

All anatomical observations were made using a Zeiss Axioskop 40 compound microscope, and images were taken using a Pixera Pro 150ES digital camera.

Table 1. Specimens examined for anatomical study.

Species	Collector & collection number	Country of origin
Badiera alternifolia (R. Rankin) J.R. Abbott	Abbott 19025	Cuba
Badiera cubensis Britton	Abbott 18894	Cuba
Badiera fuertesii Urb.	Abbott 20901	Dominican Republic
Badiera fuertesii	Abbott 21093	Dominican Republic
Badiera jamaicensis (Chod.) J.R. Abbott	Abbott 19735	Mexico
Badiera jamaicensis	Abbott 19776	Guatemala
Badiera oblongata Britton	Abbott 14363	Cuba (cultivated in Miami)
Badiera oblongata	Abbott 18900	Cuba
Badiera oblongata	Abbott 18927	Cuba
Badiera oblongata	Abbott 18977	Cuba
Badiera oblongata	Abbott 19038	Cuba
Badiera penaea (L.) DC.	Abbott 21022	Dominican Republic
Badiera penaea	Abbott 21040	Dominican Republic
Badiera penaea	Carlsward 325	Dominican Republic
Badiera propinqua Britton	Abbott 18869	Cuba
Badiera propinqua	Abbott 18871	Cuba
Badiera subrhombifolia J.R. Abbott	Abbott 20914	Dominican Republic
Badiera subrhombifolia	Skean 2500	Haiti
Badiera subrhombifolia	Skean 2419	Haiti
Badiera virgata Britton	Abbott 19034	Cuba
Badiera virgata	Abbott 19047	Cuba
Badiera virgata	Abbott 19058	Cuba
Bredemeyera lucida (Benth.) Klotzsch	Abbott 19637	Belize
Bredemeyera brevifolia (Benth.) A.W. Benn.	Abbott 16046	Bolivia
Hebecarpa barbeyana (Chodat) J.R. Abbott	Abbott 14637	USA – New Mexico
Hebecarpa macradenia (A. Gray) J.R. Abbott	Abbott 14566	USA - Texas
Hebecarpa obscura (Benth.) J.R. Abbott	Abbott 14683	USA - Texas
Polygala albicans (A.W. Benn.) Grondona	Abbott 16424	Bolivia
Polygala floribunda Benth.	Abbott 19659	Mexico

Cladistics and Character Mapping

Twelve anatomical characters from leaves (Table 2) were used to construct a character matrix (Table 3) in MacClade v.4.0.8 for Mac OS X (Maddison and Maddison, 2000). Characters were then mapped onto a modified molecular topology from Abbott (2009) using maximum parsimony. Species of *Hebecarpa* and *Polygala* were used as an outgroup, and I note that the polarity of a few characters is equivocal. Missing data were coded as ? for all analyses.

Table 2. Anatomical characters and character states mapped onto molecular-based phylogeny of *Badiera* (see Abbott, 2009). Putative pleisiomorphic states listed first (coded as 0), followed by apomorphic states (coded as 1, 2).

No.	Anatomical character and states
1.	Adaxial cuticle thickness: thick (0), thin (1).
2.	Epidermal cell shape as seen in surface view: isodiametric (0), puzzle piece shape (1).
3.	Stomatal distribution: abaxial (0), amphistomatal (1).
4.	Hair base: sunken (0), level (1), raised (2).
5.	Number of adaxial cells surrounding hair base: more than 5 (0), 5 (1).
6.	Number of abaxial cells surrounding hair base: more than 5 (0), 5 (1).
7.	Palisade distribution: only adaxial (0), adaxial and abaxial (1).
8.	Palisade seriation: 1 seriation (0), 2 seriations (1), 3 seriations (2).
9.	Palisade cell shape in upper level: height greater that 2x width (0), height less than 2x width (1).
10.	Crystal presence: absent (0), present (1).
11.	Crystal morphology: prismatic (0), druse (1).
12.	Margin: plane (0), revolute (1).

Table 3. Character state codings for *Badiera* and related genera used as outgroups. Numbers in the taxon list are collector numbers (Table 1). Missing data are coded as ?.

Taxon	1	2	3	4	5	6	7	8	9	10	11	12
Badiera alternifolia	0	0	0	1	0	0	0	2	1	1	1	1
Badiera cubensis	1	1	0	0	0	0	0	1	1	1	1	1
Badiera fuertesii 20901	0	0	0	1	?	?	0	2	1	1	1	0
Badiera fuertesii 21093	0	0	0	?	0	0	0	1	1	1	1	0
Badiera jamaicensis 19735	0	1	0	1	?	?	0	0	0	1	1	1
Badiera jamaicensis 19776	0	0	0	1	0	0	0	1	0	1	1	0
Badiera oblongata 14363	0	0	0	1	0	0	0	2	0	1	1	0
Badiera oblongata 18900	0	0	0	1	0	0	0	1	0	1	1	0
Badiera oblongata 18927	0	0	0	1	?	?	0	2	1	1	1	1
Badiera oblongata 18977	1	1	0	0	0	0	0	1	1	1	1	1
Badiera oblongata 19038	0	0	0	1	0	0	0	1	0	1	1	1
Badiera penaea 21022	0	1	0	1	0	0	0	1	0	1	1	1
Badiera penaea 21040	0	1	0	1	0	0	0	1	0	1	1	1
Badiera penaea 325	0	1	0	1	?	?	0	2	0	1	1	1
Badiera propinqua 18869	0	0	0	0	?	?	0	2	1	1	1	0
Badiera propinqua 18871	0	0	0	1	0	0	0	1	0	1	1	0
Badiera subrhombifolia 20914	0	0	0	2	?	?	0	1	0	1	1	1
Badiera subrhombifolia 2500	0	0	0	1	0	0	0	2	1	1	1	1
Badiera subrhombifolia 2419	0	0	0	1	0	0	0	2	0	1	1	1
Badiera virgata 19034	1	1	0	2	?	?	0	1	1	1	1	1
Badiera virgata 19047	0	0	0	1	0	0	0	1	0	1	1	1
Badiera virgata 19058	0	1	0	1	0	0	0	1	0	1	1	0
Bredemeyera lucida	0	0	0	0	0	0	0	0	0	1	0	0
Bredemeyera brevifolia	1	0	0	2	0	?	0	0	1	1	1	1
Hebecarpa barbeyana	1	0	1	1	0	0	1	0	0	0	?	0
Hebecarpa macradenia	1	0	1	1	1	0	1	1	0	1	1	0
Hebecarpa obscura	1	0	1	1	0	1	1	0	0	0	?	0
Polygala albicans	0	0	0	?	?	?	0	2	1	1	0	0
Polygala floribunda	1	0	0	0	1	1	0	0	0	1	1	1

RESULTS

Rehydration techniques

Compared to the control (Figs. 1a-b), samples treated in ammonium hydroxide and potassium hydroxide were less deformed and cells returned to their original turgor (Figs. 1c-f). Samples treated in water with Aerosol OT were the most deformed (Figs. 1g-h). However, cell contents remained intact in samples treated in ammonium hydroxide as well as in water with Aerosol OT (Figs. 1c-d and 1g-h). Cell contents were absent in samples treated in potassium hydroxide (Figs. 1e-f).

Leaf Anatomy of *Badiera* and Related Genera

Surface

Epidermis. Adaxial cells are rectangular with rounded corners in most species of *Badiera* (Fig. 2a), while *Badiera cubensis* Britton + *Badiera oblongata* (18977), *Badiera virgata* (19034 and 19058), *Badiera jamaicensis* (Chod.) J.R. Abbott (19735), and all *Badiera penaea* (L.) DC. have interlocking or "puzzle piece" shaped cells (Fig. 2b). Abaxial cells are also rectangular with rounded corners in most species of *Badiera*, with the exception of *Badiera cubensis, Badiera oblongata* (18977), *Badiera virgata* (19047), and *Badiera penaea* (21040). These four have epidermal cells that are interlocking. *Hebecarpa* adaxial cells are rectangular with rounded corners.

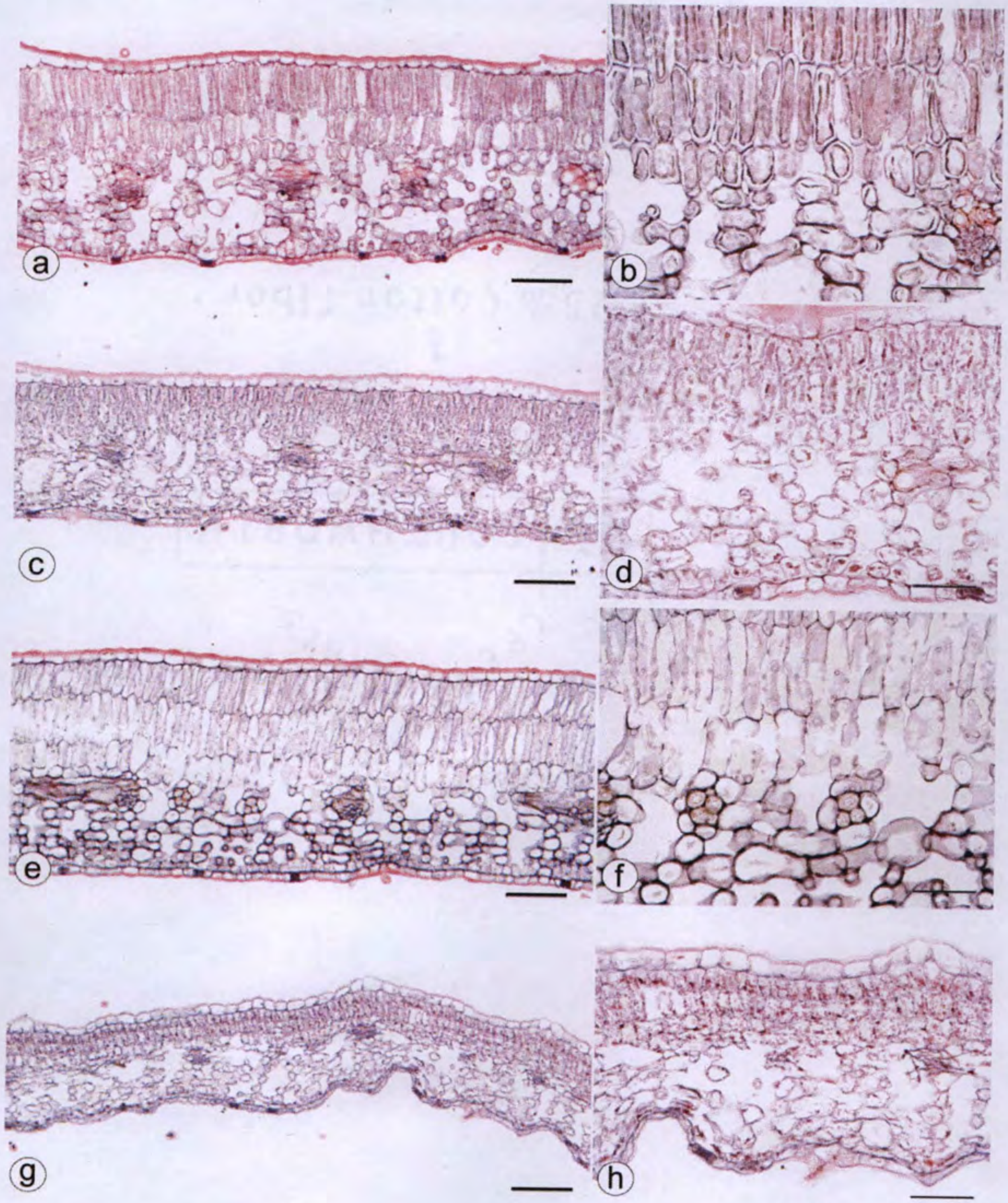

Figure 1. Transverse sections of leaves along the lamina of *Badiera oblongata*.
a) Control fixed in FAA. b) Control fixed in FAA. c) Ammonium hydroxide treatment. d) Ammonium hydroxide treatment. e) Potassium hydroxide treatment. f) Potassium hydroxide treatment. g) Water with Aerosol OT treatment. h) Water with Aerosol OT treatment. Scale bars for a, c, e, and g = 100 μm. Scale bars for b, d, f, and h = 50 μm.

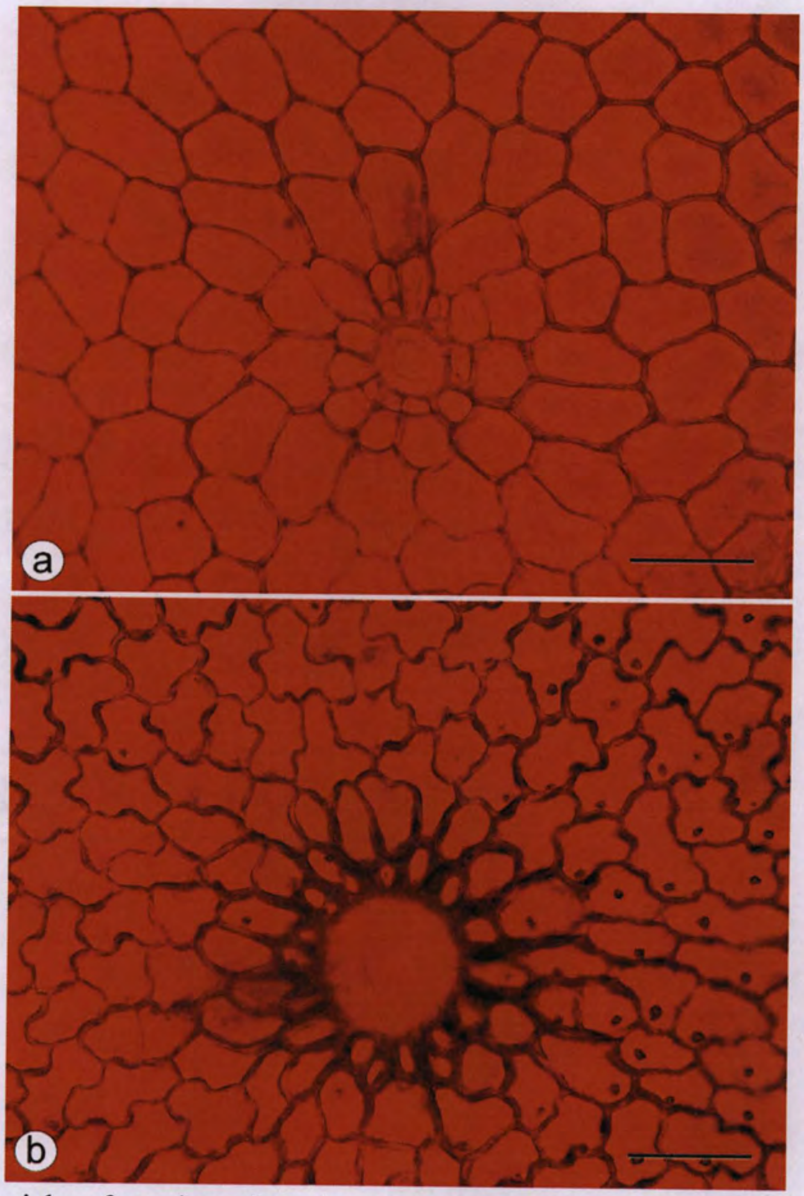

Figure 2. Adaxial surface view. a) *Badiera jamaicensis* (19776). b) *Badiera penaea* (21040). Scale bars = 50 µm.

<u>Stomata</u>. Abaxial only in *Badiera* and amphisomatal in *Hebecarpa,* while anomocytic in both.

Section

<u>Cuticle</u>. Thick and smooth except in *Badiera cubensis* (18894), *Badiera oblongata* (18977), and *Badiera virgata* (19034) where it is thin and smooth. Thin and smooth in *Hebecarpa*.

Hairs. Unicellular with the hair base even with the proximal edge of the epidermal cells (Fig. 3a). Hair base is sunken below the proximal edge of the epidermal cells (Fig. 3b) in *Badiera cubensis*, *Badiera oblongata* (18977) and *Badiera propinqua* Britton (18869) and raised (Fig. 3c) in *Badiera virgata* (19034) and *Badiera subrhombifolia* (20914). Hair base is even with proximal edge of the epidermal cells in *Hebecarpa.*

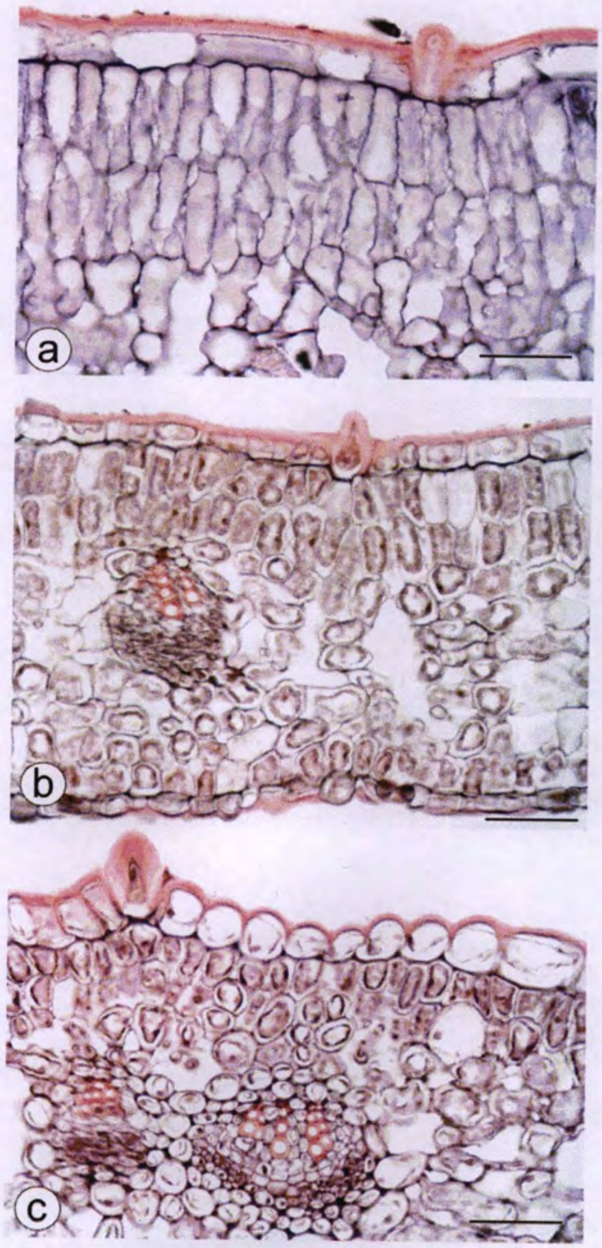

Figure 3. Hair base relative to proximal edge of epidermal cells. a) *Badiera oblongata* (19038). b) *Badiera propinqua* (18869). c) *Badiera virgata* (19034). Scale bars = 50 µm.

Epidermis. Cells are uniform in size and shape, with most being wider than long in *Badiera* and *Hebecarpa*.

Stomata. Abaxial only in *Badiera* (Fig. 4a) and amphistomatal in *Hebecarpa* (Fig. 4b).

Mesophyll. Palisade mesophyll is located adaxially in *Badiera* (Fig. 4a) and is isobilateral in *Hebecarpa* (Fig. 4b). *Badiera jamaicensis* (19735), *Hebecarpa barbeyana* (Chodat) J.R. Abbott (14637), and *Hebecarpa obscura* (Benth.) J.R. Abbott (14683) have a single seriation of palisade mesophyll and are the exception (Fig. 5a), while two or more seriations occur in all other specimens (Fig 5b). Palisade cell shape is rectangular (Fig. 6a); spongy mesophyll cell shape is isodiametric (Fig. 6b). *Hebecarpa* mesophyll contains resin cavities.

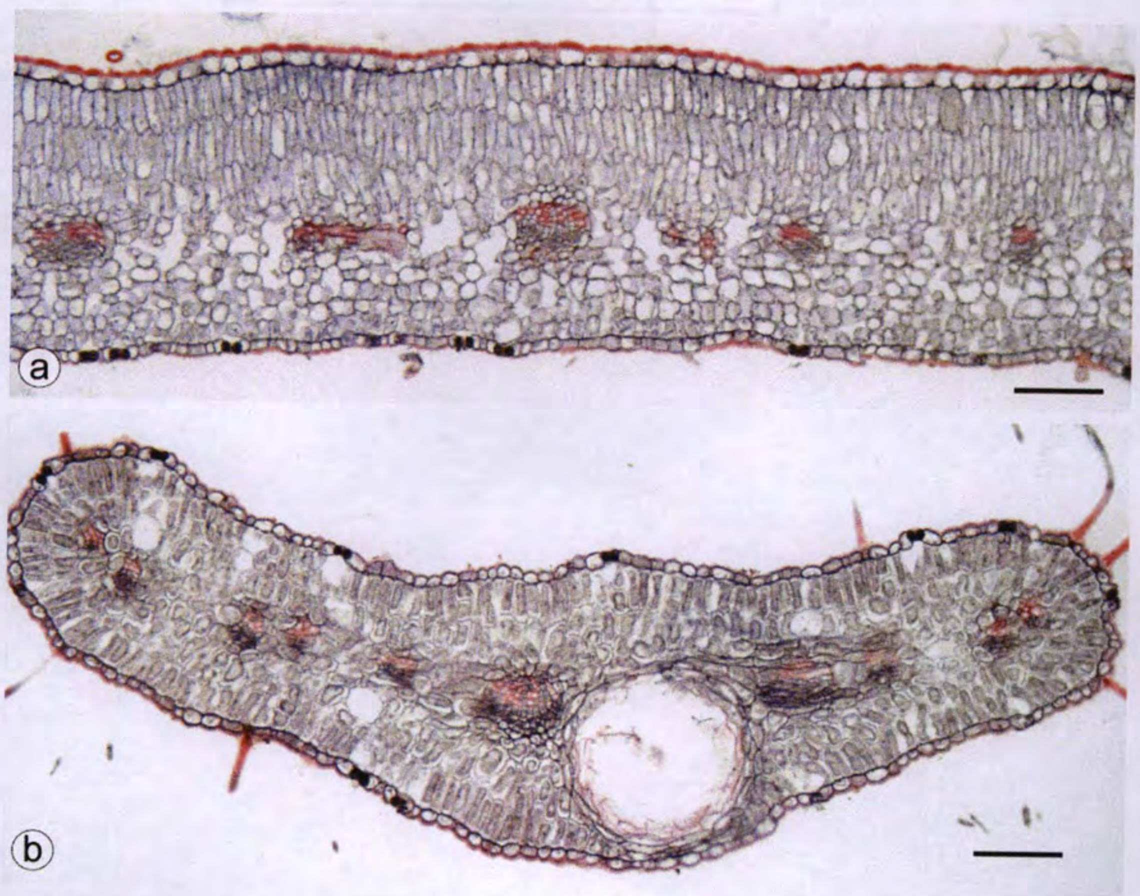

Figure 4. Stomatal and palisade distribution. a) *Badiera oblongata* (14363). b) *Hebecarpa macradenia* (A. Gray) J.R. Abbott (14566). Scale bars = 100 µm.

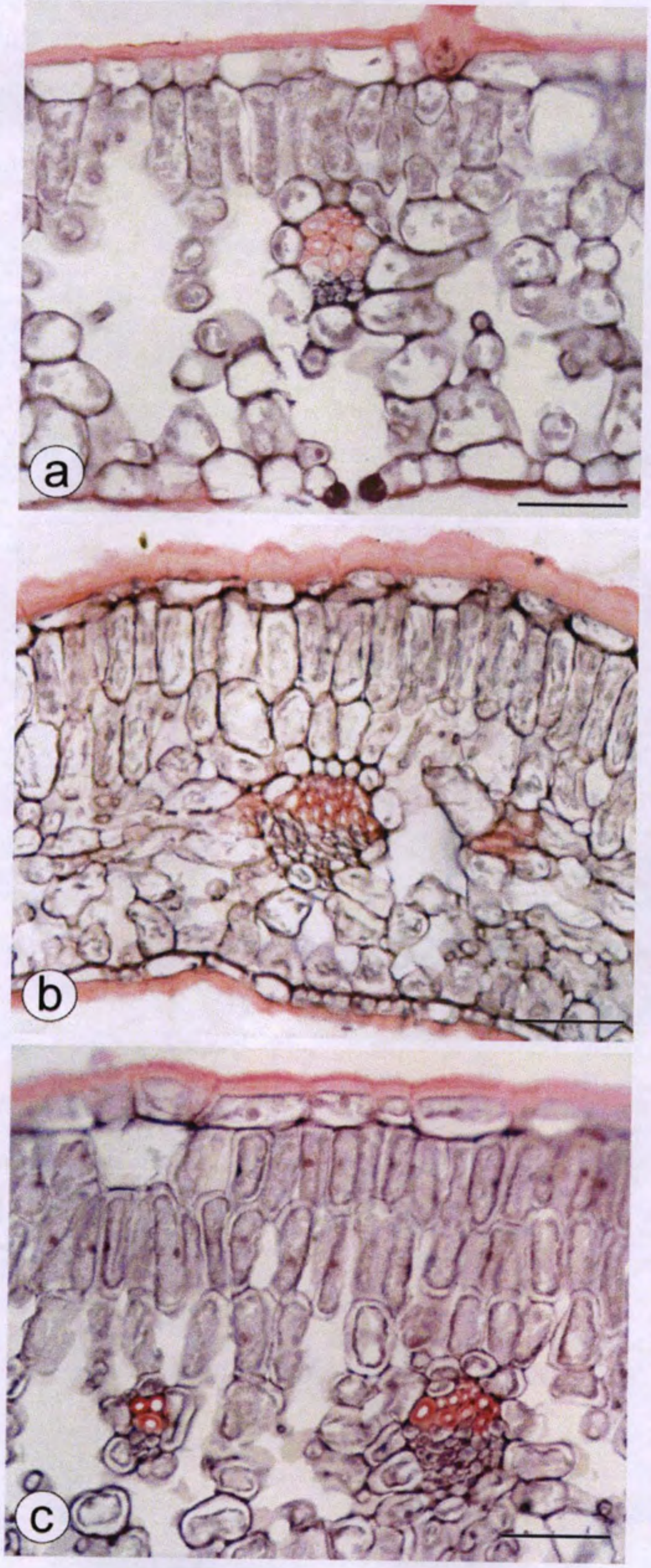

Figure 5. Palisade seriation. a) *Badiera jamaicensis* (19735). b) *Badiera virgata* (19047). c) *Badiera oblongata* (18927). Scale bars = 50 μm.

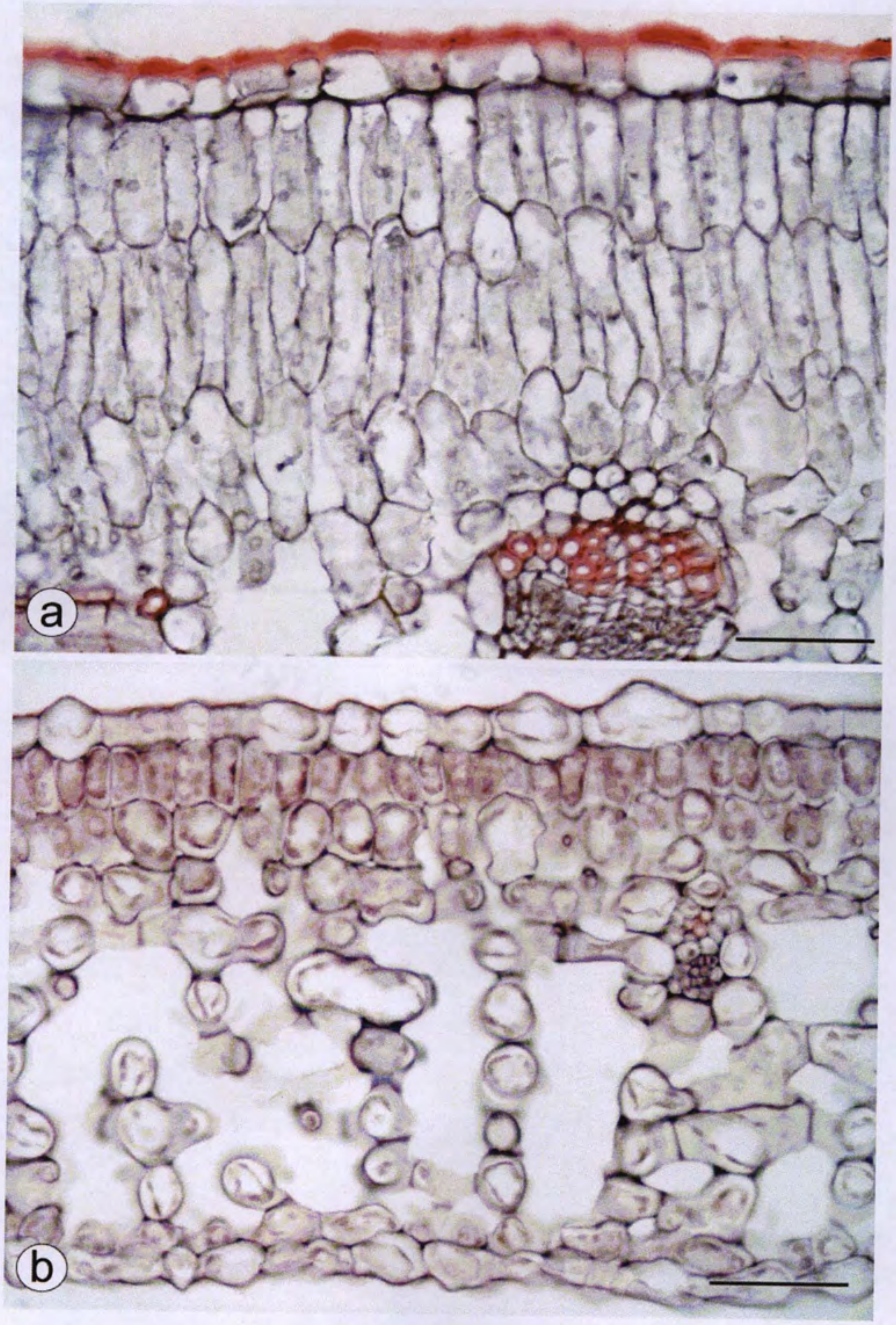

Figure 6. Mesophyll cell shapes. a) *Badiera oblongata* (14363). b) *Badiera cubensis* (18894). Scale bars = 50 μm.

Margins in section revolute (Fig. 7a) in all species of *Badiera* except *Badiera propinqua, Badiera oblongata* (14363 and 18900), *Badiera virgata* (19058), *Badiera jamaicensis* (19776), and *Badiera fuertesii* where the margin is plane in section (Fig. 7b). The margin in *Hebecarpa* is plane.

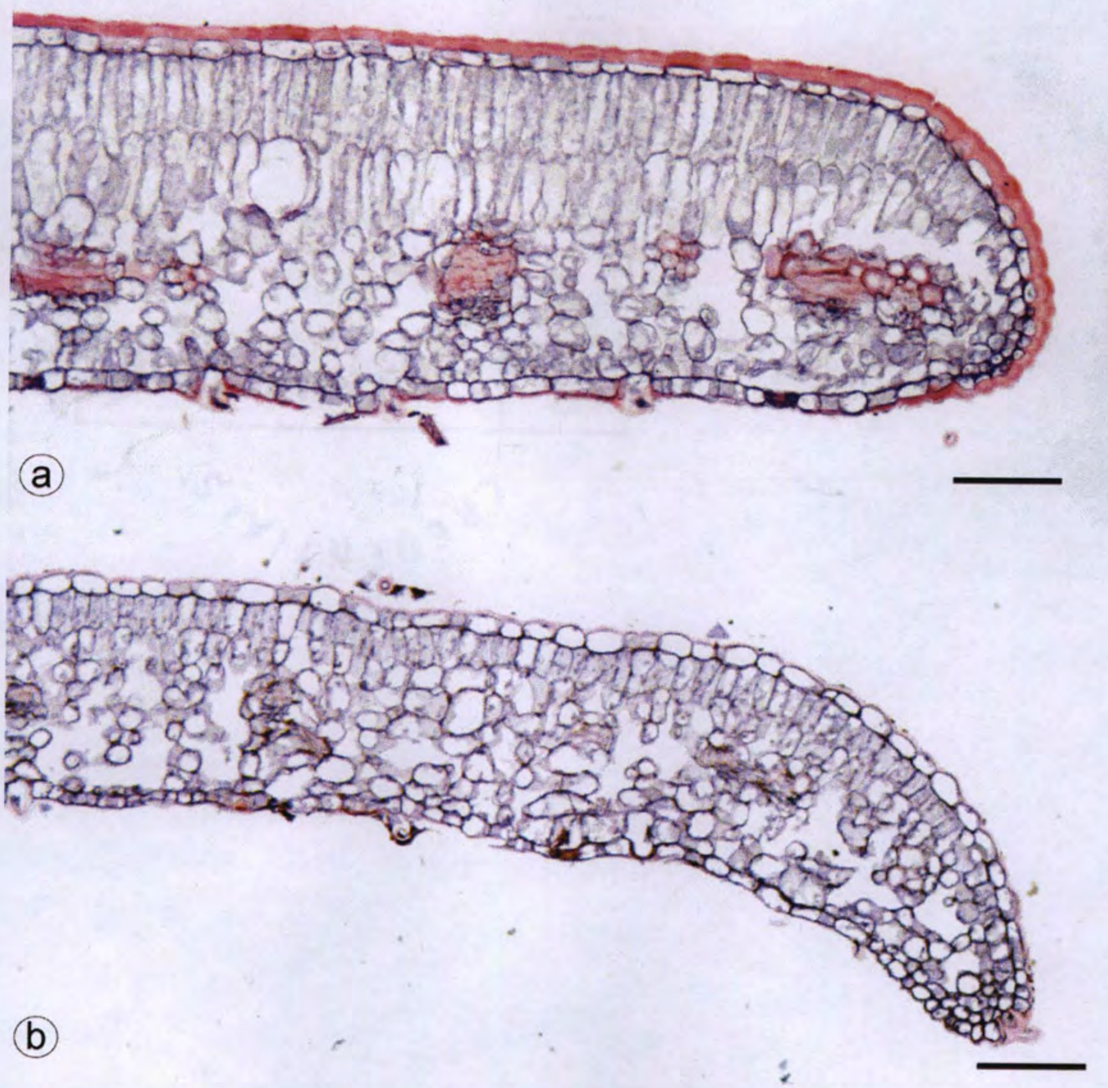

Figure 7. Margin shape in section. a) *Badiera jamaicensis* (19776). b) *Badiera oblongata* (18977). Scale bars = 100 μm.

Crystals. Druse crystals are present in all *Badiera* and in *Hebecarpa macradenia.* All other species of *Hebecarpa* lack crystals.

Vascular bundles. Collateral, with primary xylem adaxial and primary phloem abaxial, in all specimens examined.

Cladistics and Character Mapping

Badiera is defined by having a thick cuticle, with reversals in the clade *Badiera cubensis* + *Badiera oblongata* (18977) and in *Badiera virgata* (19034) (Fig. 8). *Badiera cubensis/Badiera penaea* are defined by epidermal cells that are interlocking or have a "puzzle piece" shape in surface view, with reversals in *Badiera propinqua, Badiera oblongata* (14363, 18900, 19038), *Badiera virgata* (19047), *Badiera oblongata* (18927), and *Badiera jamaicensis* (19776) (Fig. 9).

Hebecarpa is defined by leaves that are amphistomatal (Fig. 10). The clade of *Hebecarpa* + *Badiera* is defined by having level hair bases. In *Badiera virgata* (19034) and *Badiera subrhombifolia* (20914) the hair bases are raised, while the (Fig. 11) clade of *Badiera cubensis/Badiera propinqua* is defined by a reversal to sunken hair bases. The number of cells surrounding the hair base in both adaxial (Fig. 12) and abaxial (Fig. 13) epidermis is not phylogenetically informative.

Badiera retains the ancestral character of having only adaxial palisade distribution, while *Hebecarpa* is defined by isobilateral palisade distribution (Fig. 14). *Hebecarpa* + *Badiera* are unified by having a biseriate palisade, with three or more seriations occurring in *Badiera propinqua* (18869), *Badiera oblongata* (14363), *Badiera oblongata* (18927), *Badiera penaea* (C325), *Badiera subrhombifolia* (S2500)/*Badiera alternifolia* (R. Rankin) J.R. Abbott, and *Badiera fuertesii* (20901) (Fig. 15).

Clades *Badiera cubensis/Badiera propinqua* (18871), *Badiera virgata* (19034)/*Badiera oblongata* (18927), *Badiera subrhombifolia* (S2500)/*Badiera subrhombifolia* (20914) are defined by the cell shape of the upper most palisade layer being shorter than twice the cell width (Fig. 16). However, there are reversals to the ancestral state of cell heights being 2x longer than their width in *Badiera propinqua* (18871), *Badiera subrhombifolia* (S2419), and *Badiera subrhombifolia* (20914).

The clade of *Hebecarpa barbeyana* + *Hebecarpa obscura* is defined by the absence of crystals (Fig. 17). Crystal morphology is not phylogenetically informative, as most species have druses of calcium oxalate (Fig. 18).

Badiera is also defined by having revolute margins with reversals to a plane margin in *Badiera cubensis/Badiera oblongata* (18900), *Badiera virgata* (19058), *Badiera jamaicensis* (19776), and *Badiera fuertesii* (Fig. 19).

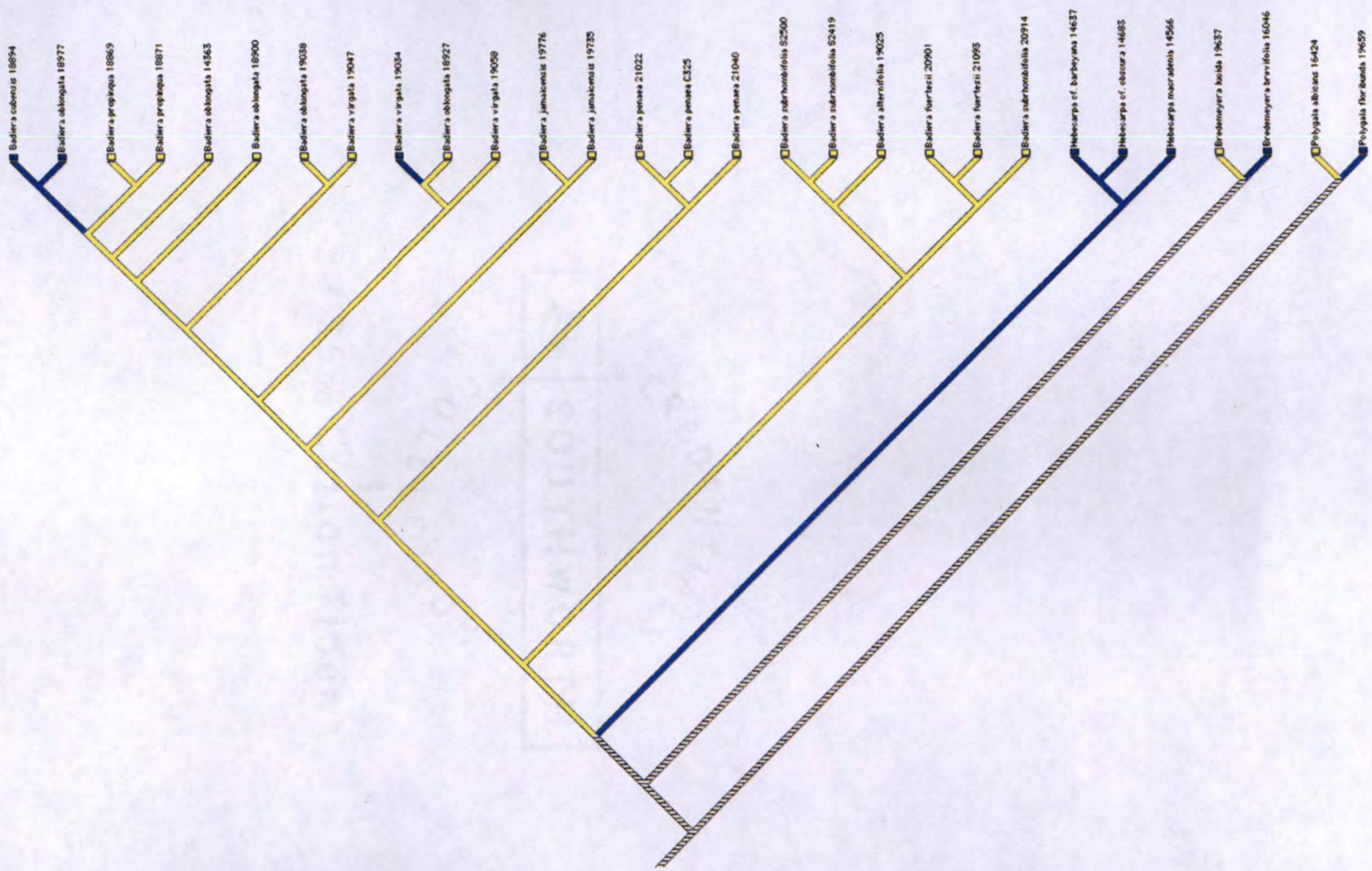

Figure 8. Character 1: adaxial cuticle thickness. Yellow represents thick cuticle (0), dark blue represents thin cuticle (1), hatched lines represent ambiguous character reconstruction.

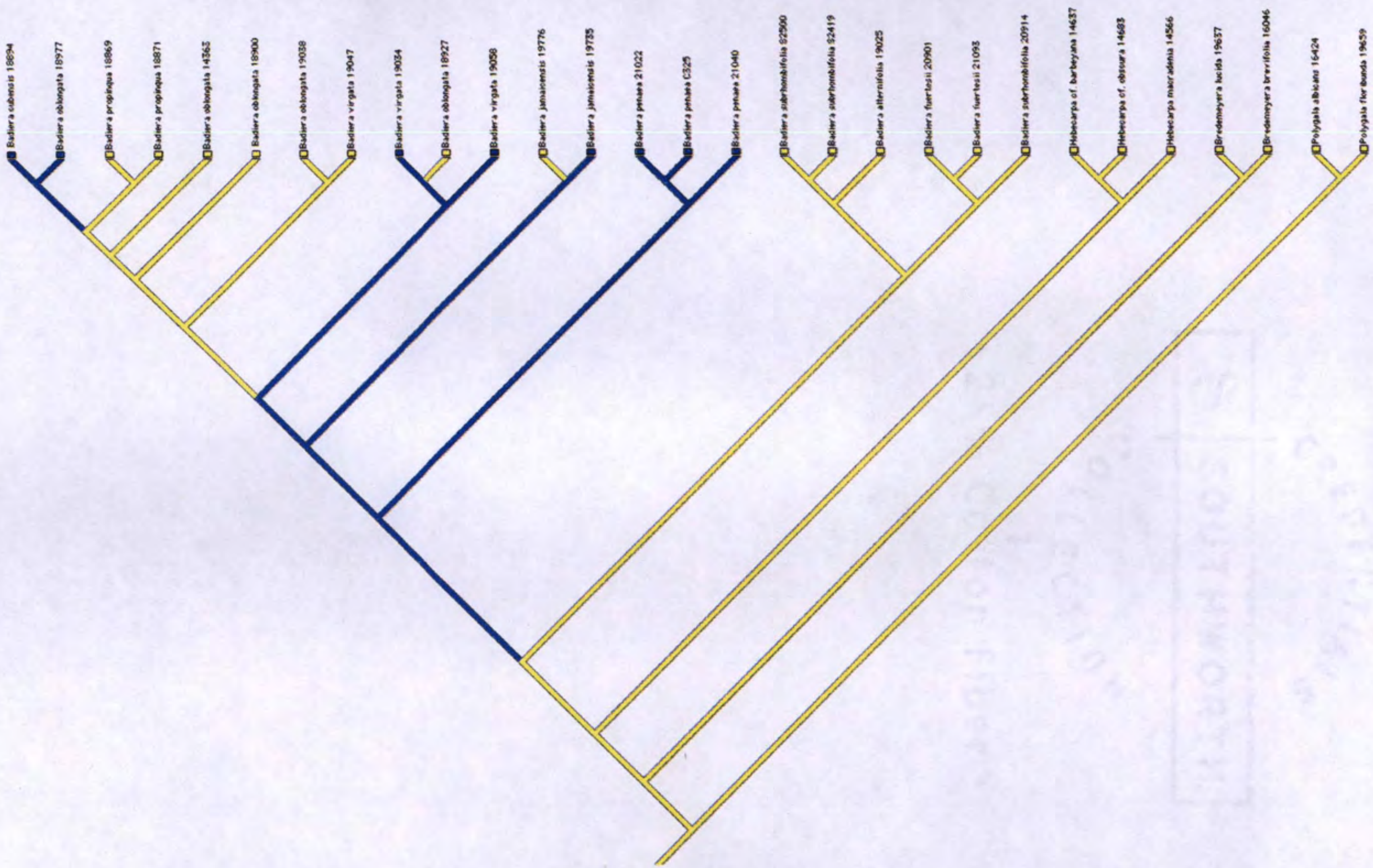

Figure 9. Character 2: epidermal cell shape in surface view. Yellow represents isodiametric (0), blue represents puzzle piece shape (1).

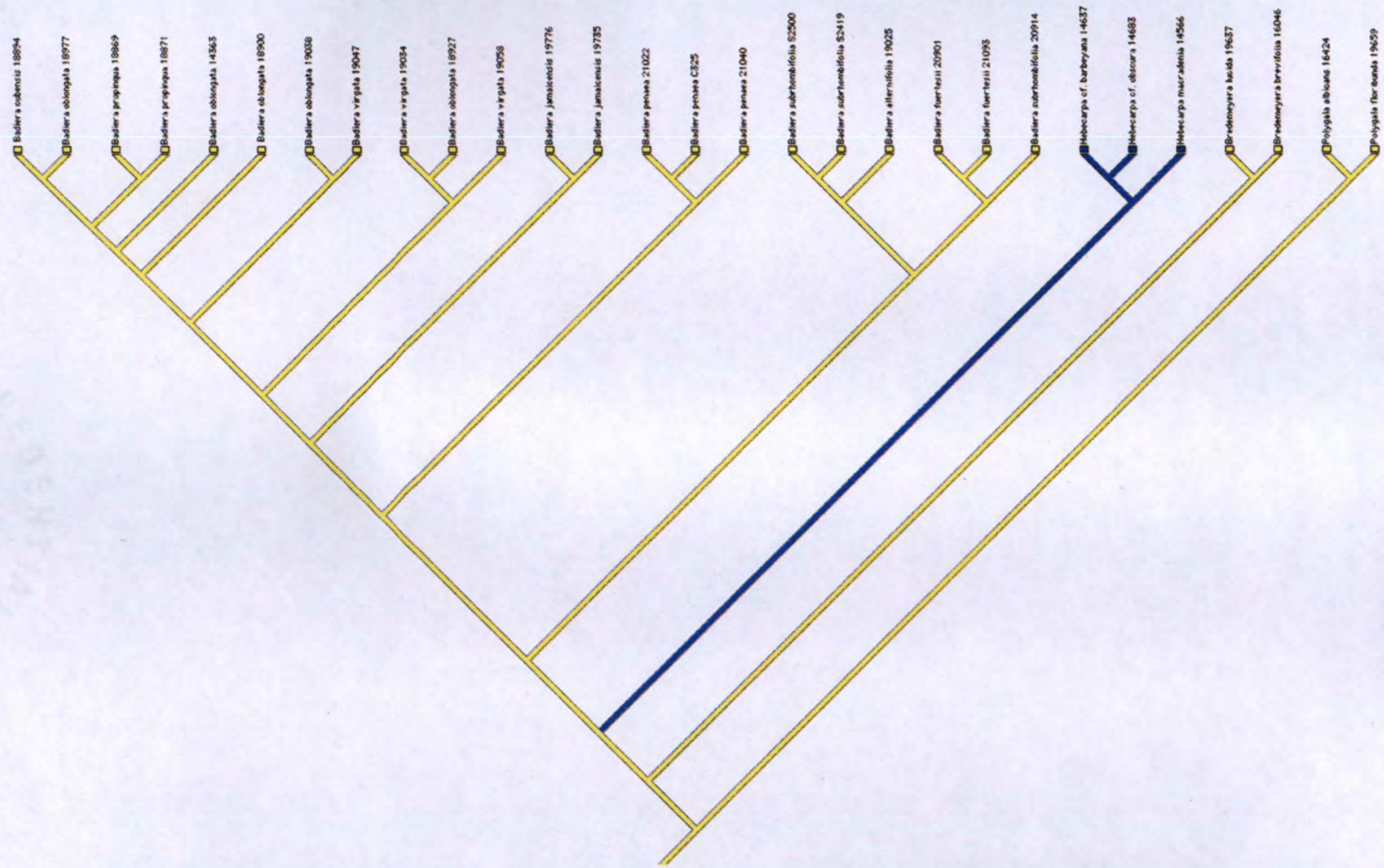

Figure 10. Character 3: stomatal distribution. Yellow represents abaxial (0), blue represents amphistomatal distribution (1).

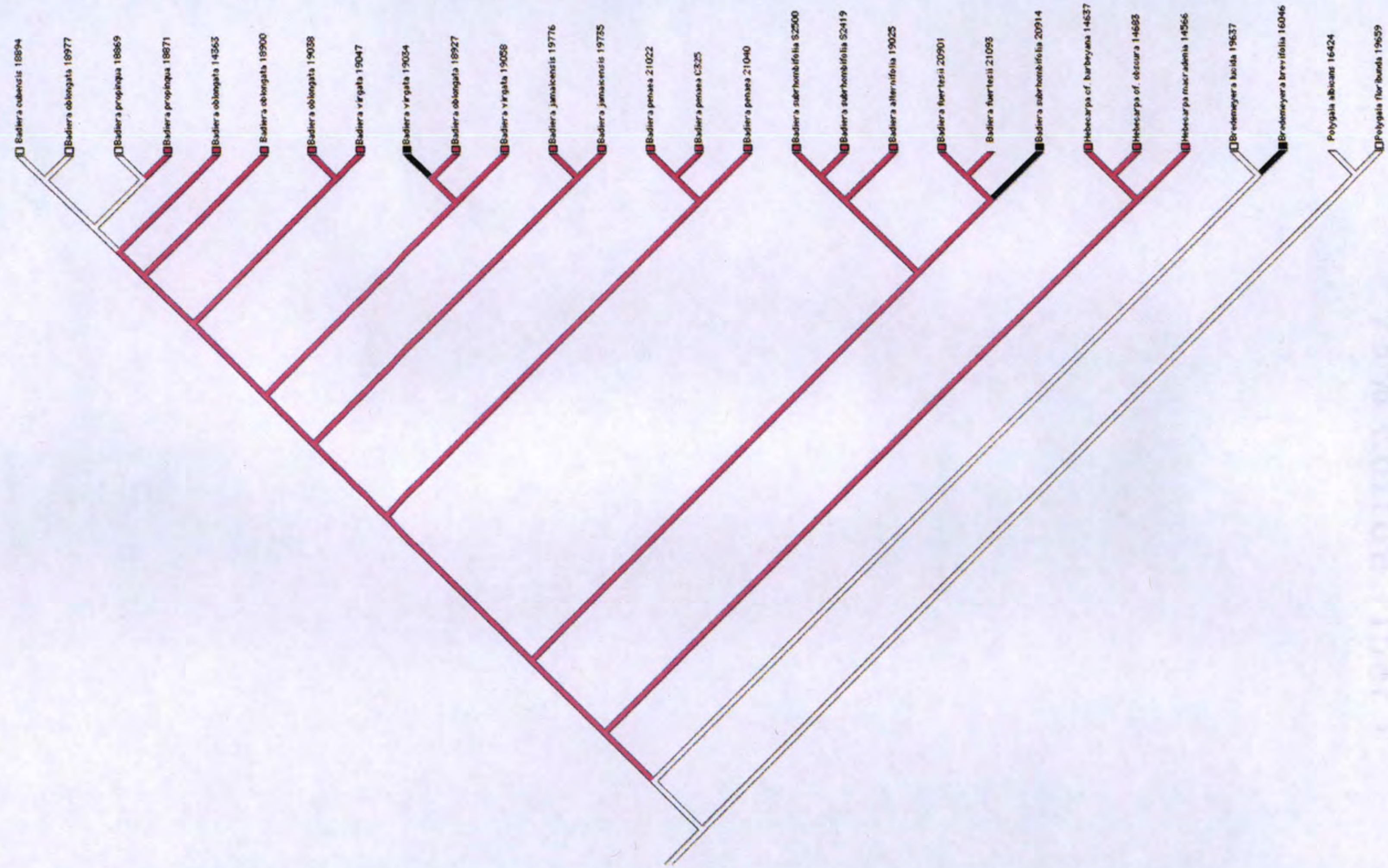

Figure 11. Character 4: hair base in relationship to proximal edge of epidermal cells. White represents sunken (0), pink represents level (1), black represents raised hair bases (2). Taxa with missing data are represented by the absence of a box.

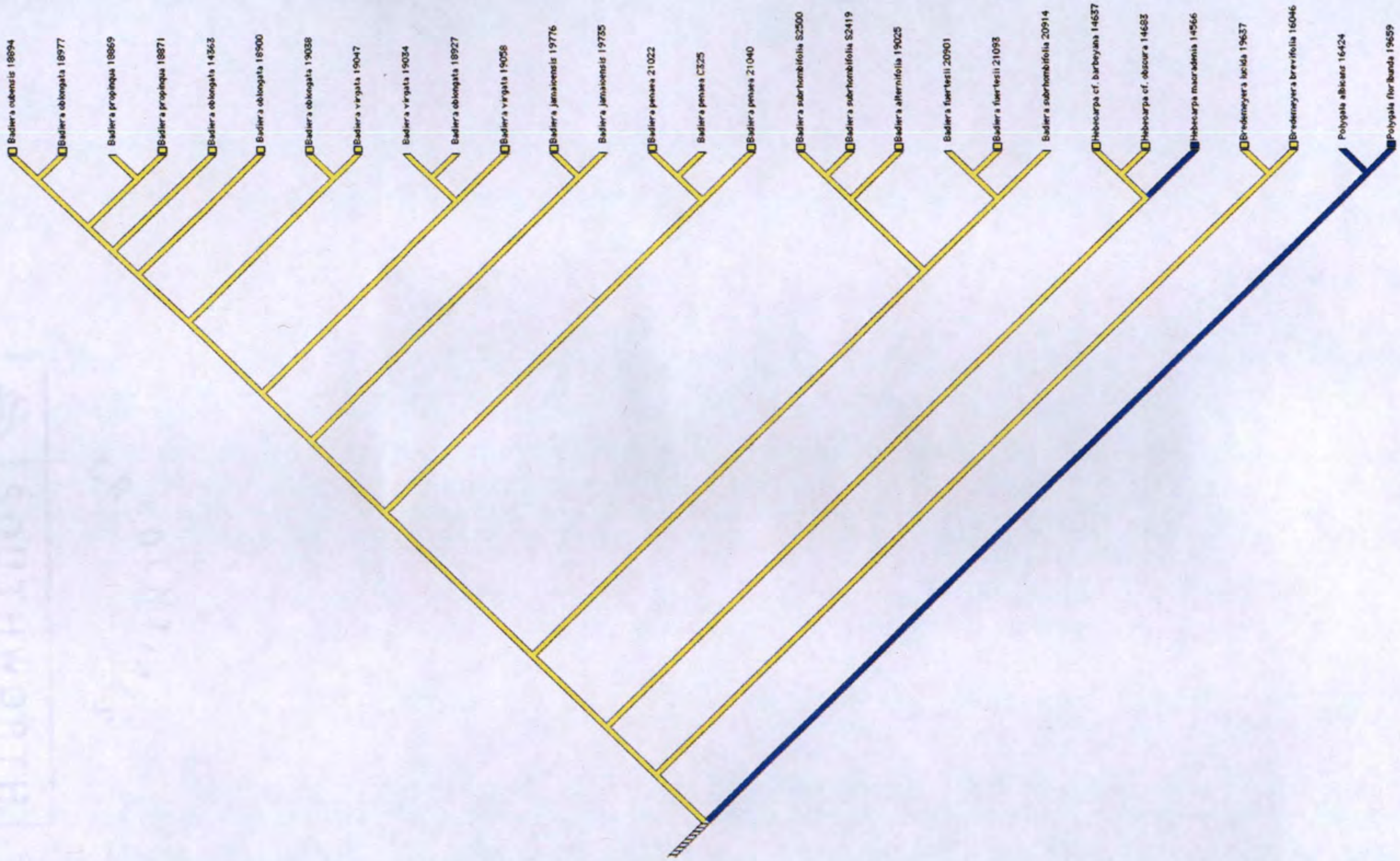

Figure 12. Character 5: number of adaxial cells surrounding hair base. Yellow represents more than 5 cells (0), blue represents 5 cells (1). Taxa with missing data are represented by the absence of a box.

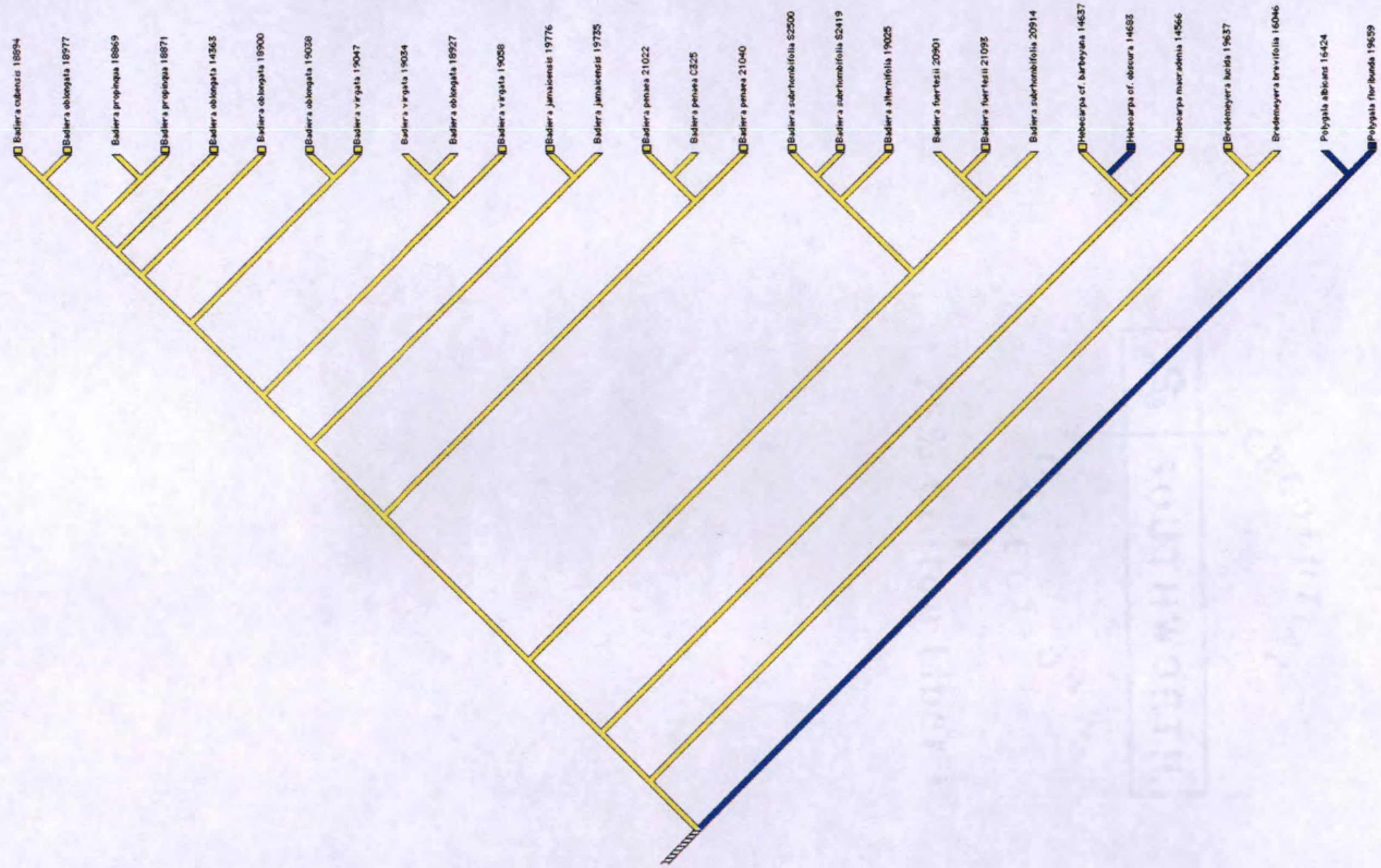

Figure 13. Character 6: number of abaxial cells surrounding hair base. Yellow represents more than 5 cells (0), blue represents 5 cells (1). Taxa with missing data are represented by the absence of a box.

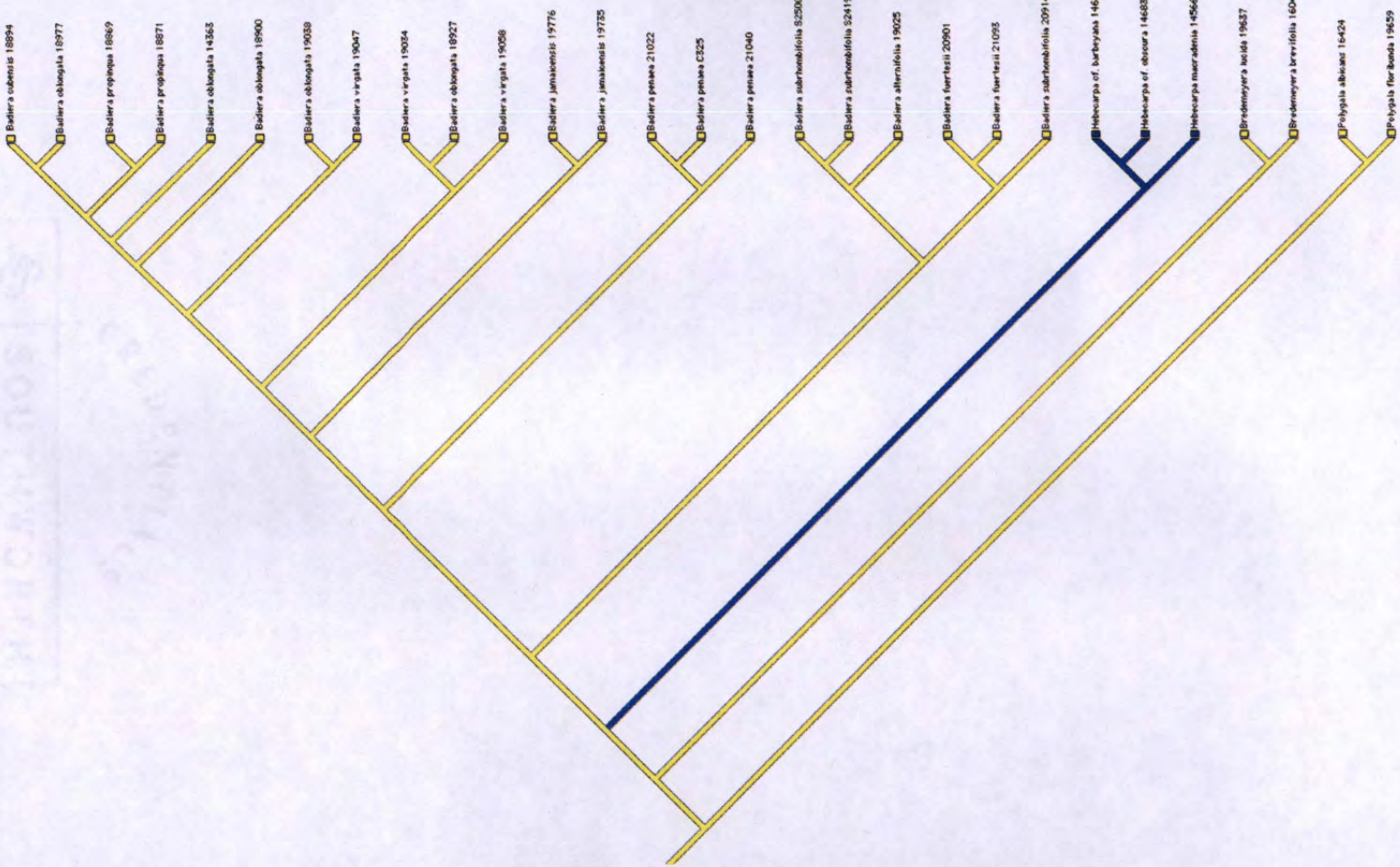

Figure 14. Character 7: palisade distribution. Yellow represents adaxial (0), blue represents isobilateral (1).

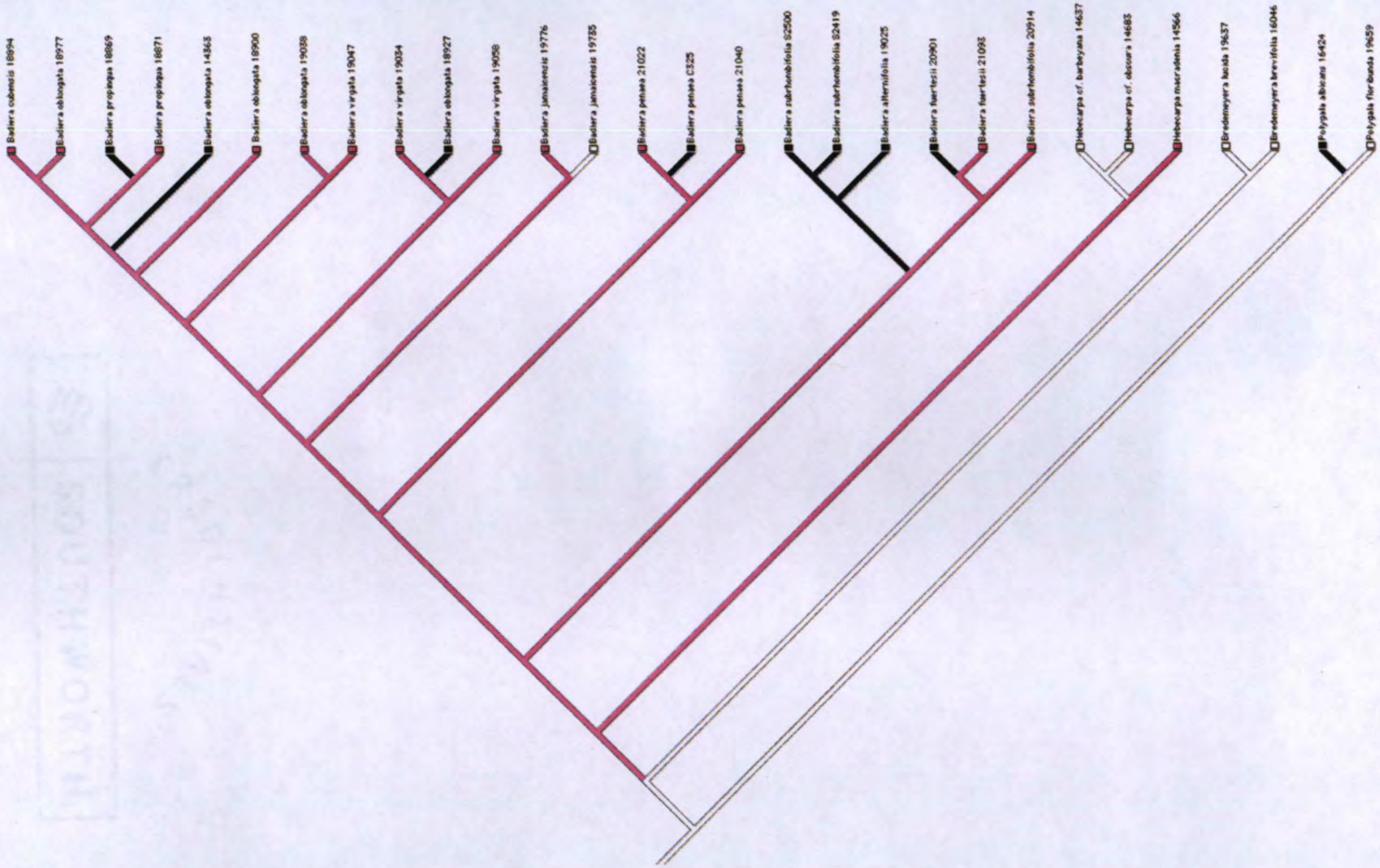

Figure 15. Character 8: palisade seriation. White represents uniseriate (0), pink represents biseriate (1), black represents 3 or more layers (2).

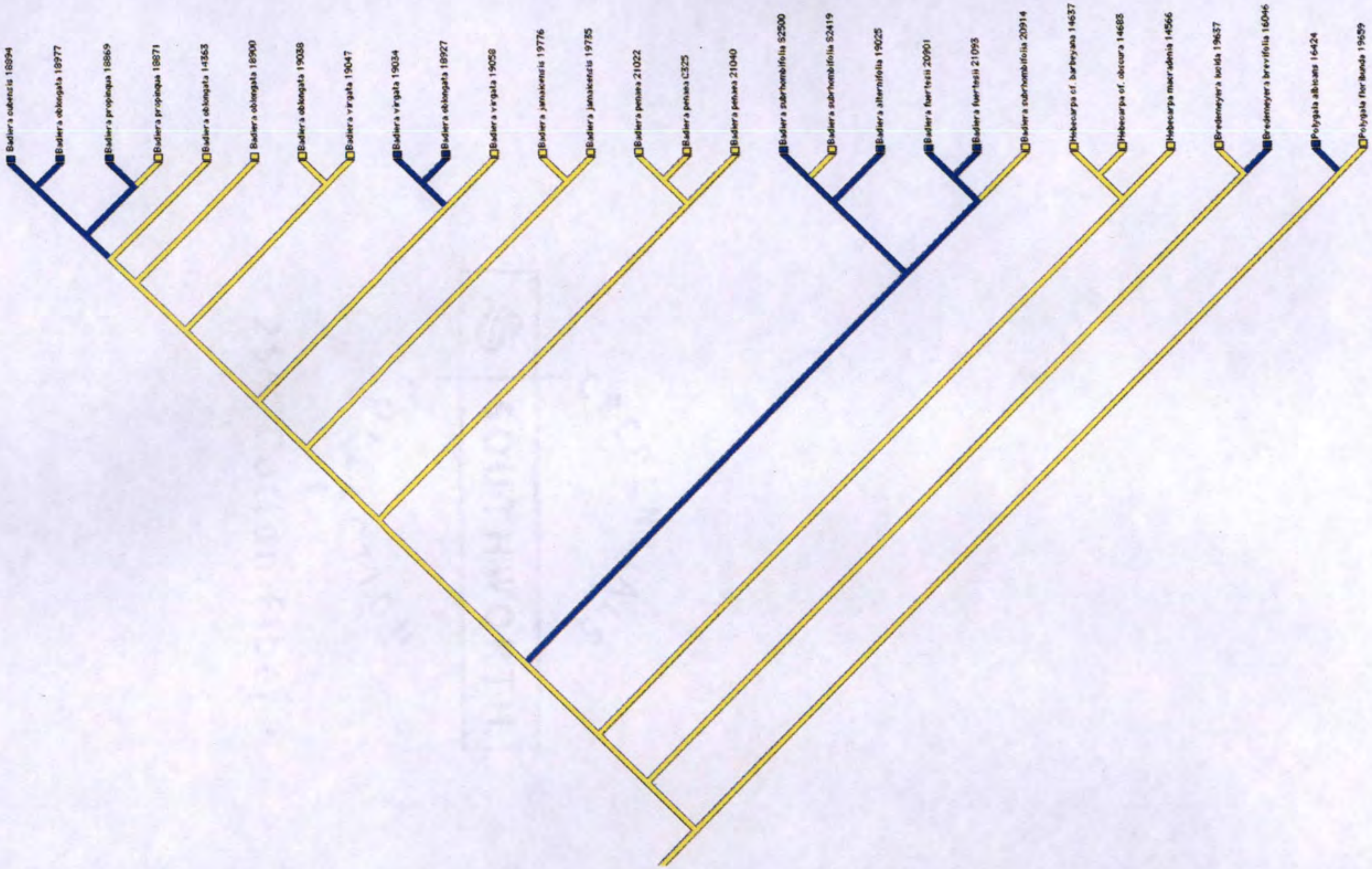

Figure 16. Character 9: palisade cell shape in upper layer as seen in cross section. Yellow represents height greater than 2x width (0), blue represents height less than 2x width (1).

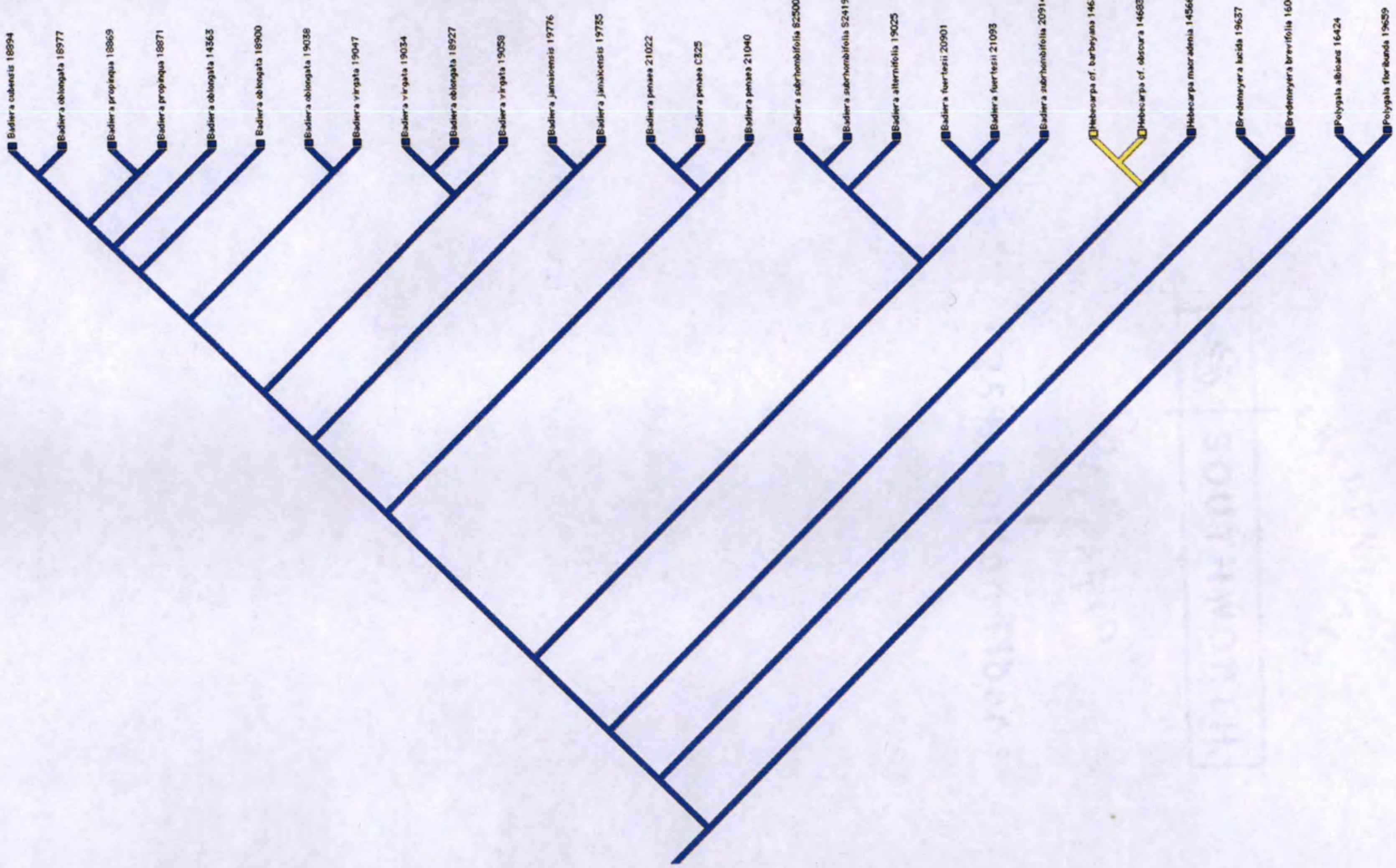

Figure 17. Character 10: crystal presence. Yellow represents absent (0), blue represents present (1).

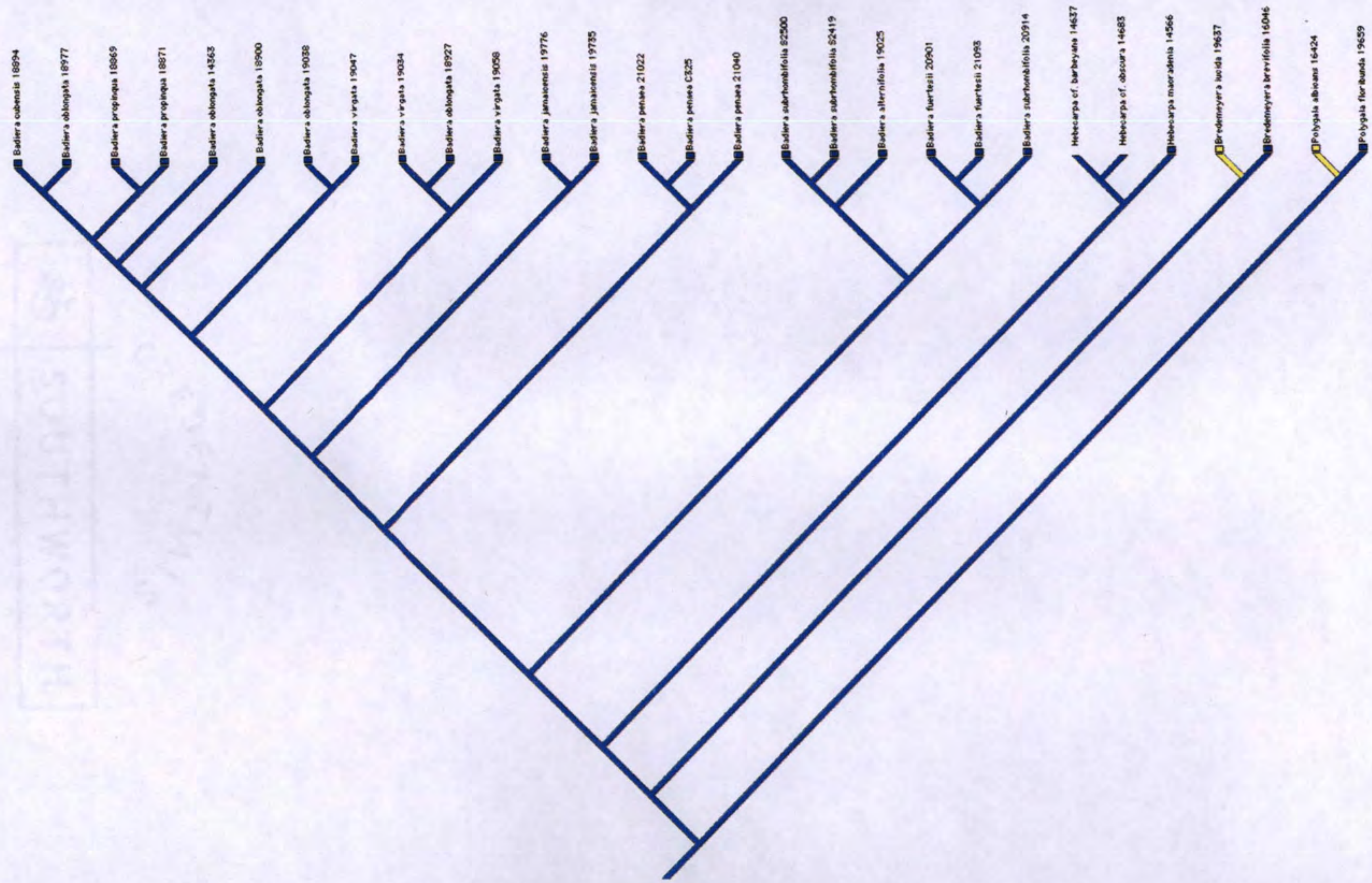

Figure 18. Character 11: crystal morphology. Yellow represents prismatic (0), blue represents druse (1). Taxa with missing data are represented by the absence of a box.

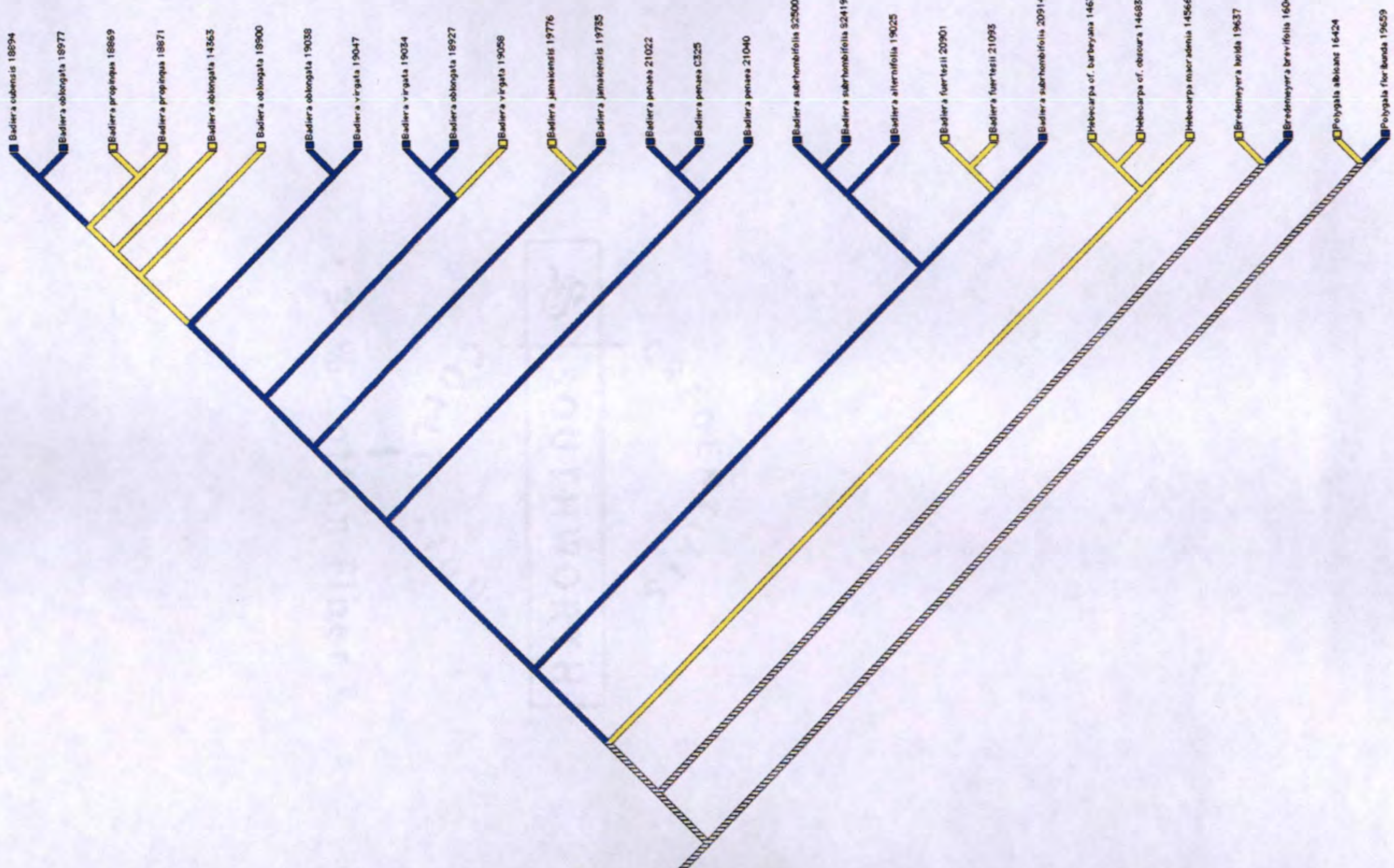

Figure 19. Character 12: leaf margin. Yellow represents plane (0), dark blue represents revolute (1), hatched lines represent ambiguous character state reconstruction.

DISCUSSION AND CONCLUSION

In research where cell shape is essential, either ammonium hydroxide or potassium hydroxide methods of rehydration are satisfactory. However, the potassium hydroxide method is more cost effective and time efficient. If cell contents are necessary, both water with Aerosol OT and concentrated ammonium hydroxide are satisfactory methods of rehydration. It should not be surprising that potassium hydroxide clears the cellular contents, as it is a similar base to sodium hydroxide, which is used in leaf clearings (Arnott, 1959). Of the methods, water with Aerosol OT is most cost effective and time efficient, so it is not surprising that this method is most commonly used in microtechnique. Rehydration of samples in ammonium hydroxide was the preferred method since it allowed for cells to return to full turgor and retains cell contents. For the characters used in this study, turgidity was important but cell contents were not.

Badiera has shared-derived characters that define it as a monophyletic genus and as a clade with the closely related *Hebecarpa*. The anatomical characters that define *Badiera* are: a thick adaxial cuticle and revolute margins. The characters that unite *Badiera* with *Hebecarpa* are: level hair bases and biseriate palisade. The ancestral character of abaxial stomatal distribution, hair bases with more than five surrounding cells, adaxial palisade distribution, palisade cells with a height greater than twice the cell width, and druse crystals are retained. Anatomical synapomorphies could potentially be adaptive to prevent water loss in these Caribbean shrubs that are constantly exposed to full sun and grow in well-drained Karst soils (Abbott and Judd, 2011).

Badiera cubensis is one of the most anatomically distinctive species in the genus. It retains several ancestral characters, including a thin cuticle and sunken hair bases.

Badiera cubensis also has several derived characters, including isodiametric adaxial epidermal shape as seen in surface view and upper layer palisade cell shape that has a height that is less than 2x the width. *Badiera cubensis* is the only specimen in this study that was collected in the understory. All other specimens grew is direct sunlight. This could account for the distinct differences found in *Badiera cubensis.*

Hebecarpa is a distinct clade united by the presence of a thin adaxial cuticle, amphistomatal leaves, isobilateral distribution of palisade mesophyll and a plane margin.

Badiera is a clade where species names, assigned by morphological studies in the late 18th and early 19th centuries, do not follow present day molecular work. Many of the anatomical characters used in this study show the same homoplasy. In terms of subgenera, the characters are not informative. Additional anatomical study of stems and roots is needed to understand structural evolution in *Badiera.*

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